CONTROLLING STARCH SYNTHESIS

FIELD OF THE INVENTION

The present invention relates to a method of breeding tomatoes with increased starch content in the young fruit and subsequently increased soluble solids content in the mature fruit. In addition, it relates to the use of genes that increase starch in the tomato.

BACKGROUND OF THE INVENTION

The solids content of ripe tomato fruit is a major determinant of its quality. Increasing the soluble solids (largely sugars and organic acids) content and thereby improving the value of industry tomatoes and the taste of fresh market tomatoes have been the goal of research projects for many years. Several approaches to improving solids levels have been taken, encompassing both agrotechnical and genetic manipulations.

Soluble solids content of tomato fruit are primarily comprised of sugars, organic acids and salts. Collectively the soluble solids content is a major determinant of fruit quality, both for industry use and for fresh market consumption. Approximately half of the soluble solids content is contributed by the sugar fraction which, in all standard cultivars of *Lycopersicon esculentum*, consists of the monosaccharide reducing sugars glucose and fructose in approximately equimolar concentrations.

Several strategies to increase sugar concentration in ripe tomato fruit have been explored. Genetic manipulations include the transfer of undefined traits of high soluble solids from wild species of Lycopersicon (Rick C.M. 1974. Hilgardia 42:493-510; and Hewitt J.D., Dinar M. and Stevens M.A. 1982. J. Am. Soc. Hort. Sci. 107:896-900) and more recently the transfer of the genetic trait of sucrose accumulation from the wild *Lycopersicon chmielewskii* (Yelle S., Hewitt J.D., Robinson N.L., Damon N.S. and Bennett A.B. 1988. Pl. Physiol. 87:737-740; and Yelle S., Chetelat R.T., Dorais M., Deverna J.W. and Bennett A.B. 1991. Pl. Physiol. 95:1026-1035.) and *L. hirsutum* (Miron D. and Schaffer A.A. 1991. Pl. Physiol. 95:623-627), as well as the transfer of the genetic trait of high fructose to glucose ratio in the mature fruit, from *L. hirsutum* (US Patent Application 08/530,216, the disclosure of which is incorporated herein by reference). The latter approach was made possible by the study of the components of carbohydrate metabolism in developing tomato fruit tissue with the purpose of identifying biochemical steps whose modification may lead to increased soluble carbohydrate content in the fruit (Yelle et al., 1988, 1991; Miron and Schaffer, 1991). Once identified, these biochemical processes could then be targeted for modification by classical genetic means, assisted by selection for the genotypic biochemical trait, or by molecular genetic strategies.

The young developing tomato fruit is characterized by a transient starch accumulation which can contribute over 25% of the dry weight of the fruit tissue. Starch concentration begins to increase within days after anthesis and reaches peak concentrations before the mature green stage (Schaffer, A.A. and Petreikov, M. 1997a. Plant Physiology 113:739-746). At the mature stage this starch is practically absent in the tomato fruit tissue. It has been hypothesized that the transiently accumulated starch serves as a reservoir of carbohydrate for the later accumulation of soluble sugars in the mature fruit (Dinar M. and Stevens M.A. 1981. J. Am. Soc. Hort. Sci. 106:415-418). Dinar and Stevens laid the groundwork for this hypothesis in their study comparing seven genotypes of tomato whose total soluble solids (TSS) values in the ripe fruit spanned the spectrum from 4.6 to 6.3 °Brix. They found that TSS values in ripe fruit were positively correlated with starch content in young, immature fruit and proposed that the products of starch hydrolysis contribute to the accumulation of soluble sugars.

The tomato plant translocates photosynthate to the fruit in the form of sucrose (Walker L.J. and Ho L.C. 1977. Ann. Bot. 41:813-823) and therefore, the temporal accumulation of starch will presumably be determined by temporal changes in the activities of key enzymes involved in sucrose to starch metabolism. The enzymatic pathway of starch synthesis in young tomato fruit has been studied and described (Schaffer, A.A. and Petreikov, M. 1997a. Plant Physiology 113:739-746; Schaffer, A.A. and Petreikov, M. 1997b. Physiologia Plantarum 101:800-806). Four enzymes were identified that potentially limit starch accumulation in these fruit, based on their absolute activities, as well as on the developmental changes in their activities which correlate temporally with the developmental changes in starch levels. These enzymes include those that catalyze the initial steps of sucrose metabolism in the young fruit (sucrose synthase, E.C. 2.4.1.13, and fructokinase, E.C. 2.7.1.4) as well as the latter steps of starch synthesis (ADP-glucose pyrophosphorylase, E.C. 2.7.7.27, and starch synthase, E.C., 2.4.1.21). In addition, Schaffer and Petreikov have shown that starch accumulation is tissue specific, localized primarily in the columella and inner pericarp tissues, and suggested that relative contributions of these tissues to fruit bulk could impact on fruit starch content.

Research has clearly shown that one of the above mentioned enzymes, ADP-glucose pyrophosphorylase (ADPGPPase), may be limiting to starch synthesis in tomato fruit, as well as in other starch accumulating tissues, such as potato tubers. In Stark D.M., Barry G.F., and Kishore G.M. 1996. Ann. NY Cad Sci 792:26-36, transgenic tomato plants and potato plants were developed with a bacterial mutant form of ADPGPPase (*E. coli*, GlgC16, a glycogen

overproducer). Transgenic tomatoes showed a higher starch content in the immature fruit and an increased sugar content in the mature fruit. Transgenic potato tubers with the same bacterial gene construct also showed an increase in starch content. Reciprocally, inhibition of ADPGPPase activity decreased the starch content of transgenic potato tubers, further indicating the importance of ADPGPPase in controlling starch accumulation.

The use of a gene for ADPGPPase of bacterial origin requires molecular genetic manipulations in order for the gene to function in eucaryotic plant tissue. For example, it requires that an artificial gene construct be developed that will encode a fusion polypeptide containing a specific amino terminal transit peptide, not present in the procaryotic gene, as well as other DNA sequence additions that will cause in plant cells transcriptional termination, and the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. In comparison, the use of a plant gene for similar transformations does not require these manipulations.

In addition, the development of plants with increased or modified activity of these enzymes, based on the natural transfer through classical breeding techniques of naturally occurring alleles of these genes, can benefit from a number of advantages. For example, classical breeding techniques lead to the positioning of the desired allele in the natural position of the gene of interest, leading to genetic stability and obviating the unpredictable "position" effects characteristic of the development of transgenic organisms. In addition, with respect to consumer preferences, there are obvious advantages of a naturally derived commercial product such as a tomato fruit, compared to a transgenically derived tomato fruit.

With respect to fructokinase, two genes from tomato fruit have been identified, cloned and sequenced (Kanayama, Y. et al. 1997. Plant Physiology 113:1379-1384). One of these genes, FK2, is particularly involved in the metabolic pathway associated with starch synthesis (Kanayama et al. 1998. Plant Physiology 117:85-90). Similarly, the gene for sucrose synthase from tomato fruit has been cloned and sequenced (Wang, F., et al. Plant Physiology 103:1463-1464;) and has been shown to be the gene for sucrose synthase of sink tissue (Fu, H. and Park, W.D. Plant Cell 7:1369-1385).

With respect to ADPGPPase, the enzyme functions in higher plants as a heterotetramer, comprised of two large and two small subunits (Preiss, J. and Sivak, M. In: Photoassimilate Distribution in Plants and Crops, Zamski, E. and Schaffer, A.A., eds., Marcel Dekker Publ, NYC, pp.63-96, 1996) which are under independent genetic control. Three separate *L. esculentum* genes coding for the large subunits and one gene for the small subunit

have recently been cloned and sequenced (Chen, B.Y. and Janes, H.,1995, Plant Physiology 109:1498; Park, S.W. and Chung, W.I. 1998. Gene 206:215-221). Much effort has been made in order to identify sources of ADPGPPase genes in plants that may contribute to improving starch content, as for example in corn (Giroux, M.J. et al., Proc. Natl. Acad. Sci. USA 93:5824-5829), where site-specific mutation of the gene for the large subunit of ADPGPPase, using a transposable element *Ds* system, led to an insertion mutation of ADPGPPase which had decreased sensitivity to the ADPGPPase inhibitor, phosphate, as well as increased seed weight.

As regards to the use of wild species of *Lycopersicon* for the modification of carbohydrate metabolism in tomatoes, as described in US Patent *Application* 08/530,216, although the fructose to glucose ratio in *L. hirsutum* is high, the actual amount of fructose and glucose is very low. Recombination of the genetic trait of fructose to glucose ratio, together with the trait of high glucose and fructose levels from *L. esculentum* yielded the unobvious and desirable trait of high levels of hexose, together with the high ratio of fructose to glucose. However, *L. hirsutum* fruit accumulate only low amounts of starch, as compared to the cultivated *L. esculentum* (Miron and Schaffer, 1991, Plant Physiology 95:623-627). Similarly, other wild species of *Lycopersicon* also accumulate little starch (i.e., *L. chmieliewskii*, Yelle et al. 1988. Plant Physiology 87:737-740). Thus, the prior art has never expected or considered the use of wild tomatoes as a possible source of genetic variability for the increase in starch accumulation.

SUMMARY OF THE INVENTION

The present invention seeks to provide selection strategies for tomatoes with high starch content in the young fruit and subsequent high soluble solids in the mature fruit.

There is thus provided in accordance with a preferred embodiment of the present invention a method for controlling starch synthesis in tomatoes including providing a population of plants derived from interspecific crosses of *Lycopersicon* spp. with *Lycopersicon esculentum* genotypes, and selecting individuals of the population that each contain an allele of a gene that increases starch synthesis, the gene originating from the *Lycopersicon* spp.

In accordance with a preferred embodiment of the present invention the step of selecting includes selecting individuals that each contain the allele of the gene that encodes for an enzyme that catalyzes a metabolic step in starch synthesis.

Further in accordance with a preferred embodiment of the present invention the step of

selecting includes selecting individuals that each contain the allele of the gene that encodes for a subunit of ADP-glucose pyrophosphorylase (ADPGPPase).

Still further in accordance with a preferred embodiment of the present invention the step of selecting includes selecting individuals that each contain the allele of the gene that encodes for a *Lycopersicon hirsutum*-derived subunit of ADPGPPase.

Additionally in accordance with a preferred embodiment of the present invention the step of selecting includes selecting by using a molecular marker for the gene.

In accordance with a preferred embodiment of the present invention the molecular marker is diagnostic for a *Lycopersicon hirsutum*-derived large subunit (LS1) of ADPGPPase.

Further in accordance with a preferred embodiment of the present invention the step of selecting includes selecting by measuring activity of the enzyme in young fruit and selecting those young fruit with high activity of the enzyme.

Still further in accordance with a preferred embodiment of the present invention the step of selecting includes selecting by measuring ADPGPPase activity of the young fruit, and selecting those young fruit with high ADPGPPase activity.

In accordance with a preferred embodiment of the present invention the *Lycopersicon* spp. includes a *Lycopersicon* spp. of green-fruited *Eriopersicon* subgenus. Preferably the *Lycopersicon* spp. includes *Lycopersicon hirsutum.*

There is also provided in accordance with a preferred embodiment of the present invention a method of producing genetically transformed plants which have elevated starch content, including the steps of inserting into the genome of a plant cell a recombinant double stranded DNA molecule including a selected promoter, a structural DNA sequence that causes the production of an RNA sequence which encodes the above described ADPGPPase LS1 protein, obtaining transformed plant cells, and regenerating from the transformed plant cells genetically transformed plants with elevated starch content.

In accordance with a preferred embodiment of the present invention the plant cell is selected from the group consisting of a tomato cell, a potato cell, a cell from a solanaceous plant, a legume cell, and a grain crop cell.

Further in accordance with a preferred embodiment of the present invention the promoter is selected from the group consisting of an immature fruit promoter, a tuber promoter, and a seed promoter.

Still further in accordance with a preferred embodiment of the present invention the step of regenerating includes regenerating genetically transformed plants with elevated starch

content in an immature fruit.

In accordance with a preferred embodiment of the present invention the step of regenerating includes regenerating genetically transformed plants with elevated starch content in a tuber.

Further in accordance with a preferred embodiment of the present invention the step of regenerating includes regenerating genetically transformed plants with elevated starch content in a seed.

Still further in accordance with a preferred embodiment of the present invention the methods of the present invention also include the step of propagating the individuals of the population or the genetically transformed plants. The propagating may be by vegetative propagation or by seed, for example.

There are also provided in accordance with a preferred embodiment of the present invention a plant produced according to any of the methods of the present invention, a fruit produced by such a plant, and a seed which when grown yields such a plant.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description, taken in conjunction with the drawing in which:

Figure 1 is a histogram of TSS (total soluble solids) values from individual plants of three BCF6 lines (95-929, 95-931 and 95-935), compared to a standard cultivar, M-82. Data from each plant is an average of TSS values from 5 individual fruit. Single plant selections from 95-929, 95-931 and 95-935 led to the BCF7 high starch breeding lines 900, 901 and 904, respectively.

In addition, the following tables are presented:

Table 1 shows the starch levels and activity of enzymes involved in the metabolism of sucrose to starch in young tomato fruit of the breeding lines 900, 901 and 904, compared to the standard cultivar, M-82. The * signifies statistical difference between each individual high starch line when compared to M-82 and does not indicate differences between the high starch lines. For the enzymes PGI (phosphoglucosisomerase), PGM (phosphoglucomutase) and UDPGPPase only one fruit was analyzed per line and since enzyme activity in all lines was relatively high and apparently in excess (as in Schaffer and Petreikov, 1997a) no significant differences were assumed. For the other assays, a minimum of 4 fruit from individual plants were assayed.

Table 2 shows the TSS values of mature fruit, and the starch levels of immature fruit

of M-82, 904, the hybrid between them, a mix of 11 hybrids between 904 and 11 introgression lines (described in text), and a mix of the 11 parallel hybrids between M-82 and the same 11 introgression lines. At least two fruit from each of the individual hybrids were measured and the average represents accordingly a minimum of 22 individual analyses. At least three fruit from each of M-82, 904 and the hybrid between them were assayed.

Table 3 shows the enzyme activities of immature fruit pericarp of M-82, 904, the hybrid between them, a mix of 6 of the 11 hybrids between 904 and 11 introgression lines (described in text), and the parallel mix of 6 of the 11 hybrids between M-82 and the same introgression lines. For M-82, 904 and the hybrid between them, two fruit from individual plants were assayed.

Table 4 shows the nucleotide sequences of the forward and reverse primers used in the PCR analysis of the 3 large and 1 small subunits of ADPGPPase and the restriction endonucleases used to digest the PCR product in order to obtain the *L. hirsutum* specific allele.

Table 5 shows the activity levels of ADPGPPase of F2 plants from the cross of line 904 and M-82. The LS1 genotype of the plants was characterized at the seedling stage, as described further herein. ADPGPPase activity and starch levels are the averages from 4 fruit (8-13 gr.) from individual F2 plants. TSS values are the average of a minimum of 5 fruit of each genotype.

Table 6 is the nucleotide sequence of ADPGlucose pyrophosphorylase, large subunit 1 (ADPGPPase LS1) from *L. hirsutum*.

Table 7 is the derived amino acid sequence for ADPGlucose pyrophosphorylase, large subunit 1 (ADPGPPase LS1) from *L. hirsutum*.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

The following is one example of carrying out the present invention. Plants of the *L. esculentum* breeding line 1630 (a Volcani Institute male sterile breeding line, used to simplify the production of the interspecific hybrid) were pollinated with pollen of the wild species *L. hirsutum* (LA1777). Hybrid F1 plants were grown and allowed to self pollinate, generating F2 seed. F2 seed were sown and about 350 plants were grown in a screenhouse and allowed to self pollinate.

Ripe fruit from each individual plant which produced fruit were individually analyzed for soluble solids (refractometrically). Only 25 of the interspecific F2 plants freely produced fruit. Pollen from one plant (F2-82) which was characterized by high soluble sugar level in the

mature fruit (71 mg soluble sugar, composed of sucrose, glucose and fructose, per gram fresh weight of fruit) was used to pollinate a standard, industry type tomato (breeding line A701) for the production of the backcross-F1 (BC-F1) population. 100 BC-F1 plants were grown in the field and mature fruit of individual plants were analyzed for soluble solids, refractometrically, as well as soluble sugars, as above. A pedigree, single seed descent selection program was carried out, selecting the plants with highest total soluble solids and soluble sugar levels. Each generation consisted of at least 100 plants. This selection technique was carried out for six generations, until the BC-F7 generation, leading to breeding lines with higher solids levels than the standard industry type cultivars.

Fig. 1 shows a series of histograms representing the BCF6 lines from which three BCF7 breeding lines were selected. The BCF6 95-929 had an average TSS value of 4.8 (11 plants, 5 fruits per plant), the BCF6 95-931 had an average TSS value of 5.7 (8 plants, 5 fruits per plant) and the BCF6 95-935 had an average TSS value of 6.1 (15 plants, 5 fruits per plant), as compared to the standard cultivar, M-82 which had an average TSS value of 3.5 (10 plants, 5 fruits per plant). The individual plant selection 95-929-6, which led to the BCF7 line 900, had a TSS of 5.5 with a plant yield of 9.1 kg fruit. The individual plant selection 95-931-2, which led to the BCF7 line 901, had a TSS of 6.5 with a plant yield of 7.2 kg fruit. The individual plant selection 95-935-5, which led to the BCF7 line 904, had a TSS of 6.6 with a plant yield of 4.7 kg fruit. The average plant yield of M-82 was 6.1 kg, based on an average of 6 plants.

In the BC-F7 generation immature fruit (approx. 15 days after anthesis) were measured for starch levels, as described in Schaffer and Petreikov (1997a). Lines 900, 901 and 904 were characterized by immature starch levels significantly higher than that of a standard industry type tomato cultivar, M-82 (Table 1). A comparative survey of enzymatic activities involved in sucrose to starch metabolism, as described in Schaffer and Petreikov (1997a), was performed on immature fruit of the two breeding lines and the standard, M-82. Typical results are presented in Table 1 and show that breeding line 900 is characterized by significantly higher levels of activity of the enzymes ADPGPPase and fructokinase while lines 901 and 904 are characterized by significantly higher activities of the enzyme ADPGPPase alone. Line 904 is characterized by the highest levels of the enzyme ADPGPPase among the lines we studied and was used for further study of the role of ADPGPPase in starch accumulation and TSS levels of tomato fruit.

The high starch line 904 was further hybridized with eleven independent tomato

breeding lines. In parallel, the standard industry type tomato cultivar, M-82, was similarly hybridized with each of these eleven lines. The eleven lines used were from the *L. pennellii* introgression lines (ILS). These introgression lines are a set of purebred lines each containing a small chromosome segment of the wild green-fruited *Lycopersicon pennellii* in the background of the cultivated *L. esculentum* cv M-82 (Eshed et al., 1992, Theor Appl. Genet., 83:1027-1034). These lines were developed from an initial interspecific cross between *L. pennellii* and *L. esculentum* cv M-82. The resulting F1 individuals were backcrossed to *L. esculentum* cv M-82 and selfed for several generations. During the process, chromosome segments of *L. pennellii* were selected for using restriction fragment length polymorphism probes covering the entire tomato genome. The introgression lines therefore provide a set of nearly-isogenic lines for segments of the wild-species genome and enable the association of yield traits with specific wild-species chromosome segments (Eshed Y. and Zamir D. 1994. Theor Appl. Genet., 88:891-897). Eleven such introgression lines were used for this study. The assumption was that crossing the 904 high starch line with this broad spectrum of genotypes, and crossing in parallel M-82 with the same identical genotypes would supply us with a broad spectrum of genetic background in which the genetic effect of 904 could be discerned.

Starch levels of the immature fruit, as well as soluble solids levels of the mature fruit, from the average of the eleven hybrids with line 904 were significantly higher than starch levels of immature fruit and soluble solids levels from mature fruit from the parallel hybrids with M-82 (Table 2). A number of these immature fruit, representing the high starch hybrids with 904 and the low starch hybrids with M-82 were subjected to a detailed enzymatic analysis of the enzymes involved in sucrose to starch metabolism in the immature tomato fruit (as described above). Table 3 shows that of the ten enzymes assayed, only ADPGPPase activity was significantly higher in the hybrids with the high starch line (904), compared to the hybrids with the M-82 line.

Table 1: Starch levels and enzyme activities of immature tomato fruit (approximately 15 DAA) for CV M-82 and three high starch breeding lines 900, 901 and 904.

	M-82	900	901	904
Starch (mg/gfw)	13.1	23.3 *	23.2 *	34.9 *
Enzymes (nmol/gfw/min)				
Invertase	15480	14690	18980	17870
Sucrose synthase	29570	31970	33260	27570
fructokinase	91	150 *	92	137
phosphoglucomutase	5760	6650	7830	7490
phosphoglucosisomerase	1950	2000	2870	2060
UDPglu PPase	15080	16760	17250	14760
ADPglu PPase	40	142 *	84 *	268 *

*Indicates statistical significance ($P < 0.05$) of each individual high starch line as compared to M-82.

Table 2: Starch content of immature fruit (approx. 15 days after anthesis) and °Brix (TSS) values of mature fruit of line 904, M-82, the hybrid between them, the mix of 11 hybrids between M-82 and 11 introgression lines (ILS) and the mix of 11 hybrids between 904 and the same 11 ILS.

Genotype	Starch mg/gfw	°Brix
M-82	23 b	4.1 b
904	58 a	8.1 a
M-82 x 904	46 a	7.1 a
M-82 x ILS	25 b	5.3 b
904 x ILS	44 a	7.5 a

Letters signify statistical significance at $P < 0.05$

Table 3: Activities of enzymes in the sucrose to starch metabolic pathway in immature tomato fruit.

	Activity (nmol/gfw/min)		
Enzyme	904 x ILS	M-82 x ILS	Ratio
Invertase	520	620	0.83
Sucrose synthase	710	560	1.27
fructokinase	225	219	1.03
glucokinase	23	25	0.94
phosphoglucomutase	6900	5340	1.31
phosphoglucoisomerase	3160	2630	1.21
UDPglu PPase	8490	7130	1.19
ADPglu PPase	190	56	3.67*
starch synthase, sol.	48	38	1.26
starch synthase, insol.	5	5	0.93

* statistical significance at $P < 0.05$

To further study the genetic trait for high ADPGPPase activity in immature fruit, specific DNA primers for the genes for the four ADPGPPase subunits (Chen and Janes, 1997 and Park and Cheung, 1998) were devised which could distinguish between the *L. hirsutum* derived gene and the *L. esculentum* derived gene, as described in the following paragraph.

PCR analysis of ADPGPPase subunits

Amplification reactions of the ADPGPPase subunits (25 µl final volume) contained 10 ng template DNA, 25 mM TAPS (pH=9.3 at 25°C), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM (mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 ng of each of the 2 primers (forward and reverse primers, see Table 4), and 1 unit of thermostable Taq DNA polymerase (SuperNova Taq polymerase, Madi Ltd., Rishon Le Zion, Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Initial incubation was at 94°C for 1 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min and 45 sec. Final polymerization at 72°C was carried out for

7 min after cycles were completed. 10 μl of the amplification products were digested with 15 units of the restriction endonuclease found to generate the *L. hirsutum* specific alleles (Table 4). Digestions were carried out according to the manufacturer recommendations (New England Biolabs Inc., Beverly, MA, USA). The digestion products were visualized by electrophoresis in 1.2% agarose gel and detected by staining with ethidum bromide.

Line 904 was shown to carry the *L. hirsutum* gene for large subunit 1 (LS1) while the other subunits of ADPGPPase in line 904 were shown to be derived from the *L. esculentum.*

In order to show that the *L. hirsutum* derived LS1 was correlated with increased ADPGPPase activity and increased starch level in the immature fruit, an F2 population of 64 plants of the cross between the high starch line 904 and the standard line M-82 was grown. The plants were genotypically typed at the first true leaf stage to determine whether they were homozygous for the *L. hirsutum* ADPGPPase LS1 allele (HH), homozygous for the *L. esculentum* allele (EE) or heterozygous (HE) containing both alleles. The 64 F2 plants segregated for the LS1 in a ratio of 16:31:17, as expected for a single locus. Immature fruit from a minimum of 4 of each of the determined F2 genotypes were assayed for starch levels and for ADPGPPase activity. Results are presented in Table 5 and clearly show that the *L. hirsutum* allele for ADPGPPase LS1, as characterized by the specific PCR primers described, is associated with increased ADPGPPase activity in the immature fruit. Furthermore, the TSS values of the mature fruit was similarly influenced by the genotype of the LS1 gene.

Table 4. Forward and reverse primers used in the PCR analysis of the 3 large and 1 small subunits of ADPGPPase and the restriction endonuclease used to digest the PCR product in order to obtain the *L. Hirsutum* specific allele.

ADPGPPase Subunit	Forward primer	Reverse primer	Restriction endonuclease
Large (LS1)	GTTCATTTGGGGA GAGTGAGCAC (Seq. ID No. 1)	GGGCAGCAGAAT TGTACTGTGTC (Seq. ID No. 2)	Hinf I
Large (LS2)	CTATTGGTGGTTG TTACCGGGT (Seq. ID No. 3)	CACTGTTCCAATA TCCTCCCAG (Seq. ID No. 4)	Hinf I
Large (LS3)	GCATATTGCTCGT GCGTACAAC (Seq. ID No. 5)	CTTTTCGCTGAAG GACATGACC (Seq. ID No. 6)	-
Small	TTTCGTCTTCTCA TCTCGCCGGA (Seq. ID No. 7)	GGCGATTTAGAG AGGCAGAGTTG (Seq. ID No. 8)	RsaI

Table 5: Effect of genotype of LS1 on ADPGPPase activity and starch levels in immature fruit and TSS in mature fruit. ADPGPPase activity and starch levels are the averages from 4 fruit (8-13 gr.) from individual F2 plants. TSS values are the average of a minimum of 5 fruit of each genotype.

Genotype	ADPGPPase	Starch	TSS
EE	104 c	16.4 b	5.3 b
EH	306 b	25.2 ab	5.9 ab
HH	450 a	37.3 a	6.3 a

Letters signify statistical difference at $P < 0.05$

Sequencing of the gene encoding ADPGPPase large subunit (LS1) from *L. hirsutum.*

Total RNA was extracted from young fruits (3 grams in weight) of an individual plant homozygous for the ADPGPPase large subunit (LS1). The RNA extraction was carried out using the TRIzol reagent system (GibcoBRL life technologies, Gaithersburg, MD, USA). The total RNA was used as template for first strand cDNA synthesis using the Superscript preamplification system (GibcoBRL life technologies, Gaithersburg, MD, USA). The cDNA prepared was used as template in a PCR reaction to amplify the gene encoding ADPGPPase large subunit (LS). The DNA fragments containing the ADPGPPase large subunit (LS) were excised from an agarose gel and purified using the GENECLEAN II kit (BIO 101 inc., La Jolla CA, USA). The PCR bands were then cloned into an pGEM-T Easy vector using the pGEM-T and pGEM-T Easy Vector Systems according to the manufacturer recommendations (Promega corporation, Madison, WI, USA). The DNA clones were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA, USA).

Table 6 is the nucleotide sequence of ADPGPPase LS1 (ADPGlucose pyrophosphorylase, large subunit 1) from *L.hirsutum* (Seq. ID No. 9). Table 7 is the derived amino acid sequence for ADPGPPase LS1 (ADPGlucose pyrophosphorylase, large subunit 1) from *L. hirsutum* (Seq. ID No. 10).

Table 6: Nucleotide sequence of ADPGPPase LS1 (ADPGlucose pyrophosphorylase, large subunit 1) from *L. hirsutum* (Seq. ID No. 9).

1 ATGAAATCGA CGGTTCATTT GGGGAGAGTG AGCACTGGTG GCTTTAACAA
51 TGGAGAGAAG GAGATTTTTG GGGAGAAGAT GAGAGGGAGT TTGAACAACA
101 ATCTCAGGAT TAATCAGTTG TCGAAAAGTT TGAAACTTGA GAAGAAGGAG
151 AAGAAGATTA AACCTGGGGT TGCTTACTCT GTGATCACTA CTGAAAATGA
201 CACAGAGACT GTGTTCGTAG ATATGCCACG TCTTGAGAGA CGCCGGGCAA
251 ATCCCAAGGA TGTGGCTGCA GTCATATTAG GAGGAGGCGA AGGGACCAAG
301 TTATTCCCAC TTACAAGTAG AACTGCAACC CCTGCTGTTC CGGTTGGAGG
351 ATGCTACAGG CTCATAGACA TCCCGATGAG CAACTGTATC AACAGTGCTA
401 TTAACAAGAT TTTTGTGCTG ACACAGTACA ATTCTGCTGC CCTGAATCGT
451 CACATTGCTC GAACGTATTT TGGCAATGGT GTGAGCTTTG GAGATGGATT
501 TGTCGAGGTA CTAGCTGCAA CTCAGACACC TGGGGAAGCA GGAAAAAAAT
551 GGTTTCAAGG AACAGCAGAT GCTGTCAGAA AATTTATATG GGTTTTTGAG
601 GACGCTAAGA ACAAGAATAT TGAAAATATC CTTGTATTAT CTGGGGATCA
651 TCTTTATAGG ATGGATTATA TGGAGTTGGT GCAGAACCAT ATTGACAGAA
701 ATGCTGATAT TACTCTTTCA TGTGCACCAG CTGAGGACAG CCGAGCATCA
751 GATTTTGGGC TGGTCAAGAT TGACAGCAGA GGCAGAGTTG TCCAGTTTGC
801 TGAAAAACCA AAAGGTTTTG AGCTTAAAGC AATGCAAGTA GATACTACTC
851 TTGTTGGATT ATCTCCACAA GATGCGAAGA AATCCCCTTA TATTGCTTCA
901 ATGGGAGTTT ATGTTTTCAA GACAGATGTA TTGCTGAAGC TCTTGAAATG
951 GAGCTACCCC ACTTCTAATG ATTTTGGCTC TGAAATTATA CCAGCAGCTA
1001 TTGATGATTA CAATGTCCAA GCATACATTT TCAAAGACTA TTGGGAGGAC
1051 ATTGGAACAA TTAAATCTTT CTATAATGCT AGCTTGGCGC TCACACAAGA
1101 GTTTCCAGAG TTCCAATTTT ATGATCCAAA AACACCTTTT TACACATCTC
1151 CTAGGTTCCT TCCACCAACC AAGATAGACA ATTGCAAGAT TAAGGATGCC
1201 ATAATTTCTC ATGGATGTTT CTTGCGAGAT TGCTCTGTGG AACACTCCAT
1251 AGTGGGTGAA AGATCACGCT TAGACTGTGG TGTTGAACTG AAGGATACTT
1301 TCATGATGGG AGCAGACTAC TACCAAACAG AATCTGAGAT TGCCTCCCTG
1351 TTAGCAGAGG GGAAAGTACC GATTGGGATT GGGGAAAATA
CAAAAATAAG
1401 GAAATGTATC ATTGACAAGA ACGCAAAGAT AGGAAAAAAT GTTTCAATCA
1451 TTAATAAAGA TGGTGTTCAA GAGGCAGACC GACCAGAGGA AGGATTCTAC
1501 ATACGATCAG GGATAACCAT TATATCAGAG AAAGCCACAA TTAGAGATGG
1551 AACAGTTATA TGA

Table 7: Derived amino acid sequence for ADPGPPase LS1 from *L. hirsutum* (Seq. ID No. 10).

MKSTVHLGRVSTGGFNNGEKEIFGEKMRGSLNNNLRINQL
SKSLKLEKKEKKIKPGVAYSVITTENDTETVFVDMPRLERRRAN
PKDVAAVILGGGEGTKLFPLTSRTATPAVPVGGCYRLIDIPMSNC
INSAINKIFVLTQYNSAALNRHIARTYFGNGVSFGDGFVEVLAAT
QTPGEAGKKWFQGTADAVRKFIWVFEDAKNKNIENILVLSGDHL
YRMDYMELVQNHIDRNADITLSCAPAEDSRASDFGLVKIDSRGR
VVQFAEKPKGFELKAMQVDTTLVGLSPQDAKKSPYIASMGVYV
FKTDVLLKLLKWSYPTSNDFGSEIIPAAIDDYNVQAYIFKDYWED
IGTIKSFYNASLALTQEFPEFQFYDPKTPFYTSPRFLPPTKIDNCKI

KDAIISHGCFLRDCSVEHSIVGERSRLDCGVELKDTFMMGADYY
QTESEIASLLAEGKVPIGIGENTKIRKCIIDKNAKIGKNVSIINKDG
VQEADRPEEGFYIRSGITIISEKATIRDGTVI

In the foregoing example, the large subunit 1 of ADPGPPase was shown to increase starch level. Although not specifically tested, it is reasonable to assume that the present invention can also be carried out by transferring the *L. hirsutum* genes for any of the other 3 subunits of the enzyme, using the specific PCR markers developed for each of these genes, as they may also increase starch. In addition, transfer of ADPGPPase genes from other wild tomato species, other than *L. hirsutum*, may also increase starch in crosses with *L. esculentum*. Additionally, transfer of genes for other enzymes of starch synthesis from wild species, such as fructokinase and sucrose synthase for which the gene sequences from *L. esculentum* are known, may also increase starch levels.

Those skilled in the art will recognize that the described gene can be used to genetically transform plants to increase starch content. Plants that can genetically be transformed to have increased starch content include a large range of agriculturally important crops, such as but not limited to, potato, tomato, corn, wheat, cotton, banana, soybean, pea and rice. The plant transformation technology, including methods of transformation, such as the use of *Agrobacterium tumefaciens*, and methods of developing constructs, including the use of tissue specific promoters is well established and has recently been reviewed by Christou, P. ("Transformation technology", Trends in Plant Science, 1:423-431). There are presently available numerous promoters, including the constitutive promoters (CaMV) 35S and the maize ubiquitin promoter. In addition, there are, for example, organ/tissue specific promoters, for expression in seeds, tubers, immature fruit, mature fruit, pollen, roots and other organs.

The above examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications can be made to the methods described herein while not departing from the spirit and scope of the present invention.